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# Human T Cell Responses to Dengue Virus Antigens

## Proliferative Responses and Interferon Gamma Production

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### Abstract

The severe complications of dengue virus infections, hemorrhagic manifestations and shock, are more commonly observed during secondary dengue virus infections than during primary infections. It has been speculated that these complications are mediated by cross-reactive host-immune responses. We have begun to analyze human T cell responses to dengue antigens in vitro to explain the possible role of T lymphocytes in the pathogenesis of these complications. Dengue antigens induce proliferative responses of PBMC from dengue antibody-positive donors, but do not induce specific proliferative responses of PBMC from dengue antibody-negative donors. IFN $\gamma$  is detected in the culture fluids of dengue-immune PBMC stimulated with dengue antigens. The cells that proliferate in the dengue antigen-stimulated bulk cultures have CD3+, CD4+, CD8-, CD16-, and CD20- phenotypes. Dengue-specific T cell lines were established using limiting dilution techniques. They have CD3+, CD4+, and CD8- phenotypes, and produce IFN $\gamma$  in response to dengue antigens. Culture fluids from dengue-immune PBMC stimulated with dengue antigens, which contain IFN $\gamma$ , augment dengue virus infection of human monocytes by dengue virus-antibody complexes.

These results indicate that PBMC from dengue-immune donors contain CD4+ T cells that proliferate and produce IFN $\gamma$  after stimulation with dengue antigens, and suggest that the IFN $\gamma$  that is produced by these stimulated dengue-specific T cells may contribute to the pathogenesis of dengue hemorrhagic fever and dengue shock syndrome by increasing the number of dengue virus-infected monocytes in the presence of cross-reactive anti-dengue antibodies.

### Introduction

The severe complications of dengue virus infections, hemorrhagic manifestations and shock, are much more commonly observed during secondary infections with a heterologous serotype of dengue virus than during primary infections (1). It

has been speculated that dengue hemorrhagic fever (DHF)<sup>1</sup> and dengue shock syndrome (DSS) may be mediated by cross-reactive host-immune responses (2). Halstead et al. have reported that anti-dengue antibodies at subneutralizing concentrations enhance dengue virus infection of Fc receptor-positive cells (3, 4). They have hypothesized that the number of dengue virus-infected monocytes increases in secondary infections because of the presence of anti-dengue antibodies and this results in DHF and DSS (1).

We recently reported that IFN $\gamma$  augments dengue virus infection of human monocytes and a human monocytic cell line, U937 (5). This augmentation required the presence of anti-dengue antibody, and the augmentation of infection was significantly correlated with the increased expression of Fc $\gamma$  receptors induced by IFN $\gamma$ . We reported earlier that PBMC from influenza-immune donors produce high titers of IFN $\gamma$  when stimulated with influenza virus in vitro (6). These two observations stimulated us to consider further the role of dengue-specific T cells in the pathogenesis of dengue virus infections. In this paper we describe human T cell proliferative responses and IFN $\gamma$  production in vitro induced by dengue antigens. PBMC from dengue antibody-positive donors proliferate and produce IFN $\gamma$  after stimulation with dengue antigens. The responding cells are T cells (CD3+) with CD4 (helper/inducer) phenotype. The IFN $\gamma$  produced augments dengue virus infection of human monocytes by dengue virus-antibody complexes.

### Methods

**Human PBMC.** Peripheral blood specimens were obtained from healthy adult Thai Red Cross blood bank donors in Bangkok, Thailand, and from two American donors who were known to have been infected with dengue virus. The sera of these donors contained hemagglutination-inhibiting (HAI) antibodies to dengue viruses (mean HAI titers; 1:49 to dengue 1, 1:58 to dengue 2, 1:118 to dengue 3, 1:272 to dengue 4). Most of the Thai people have antibodies to dengue viruses by age 10 (7). The two American donors had previously been immunized with yellow fever vaccine. Both were known to have been infected with dengue 3 virus, strain CH53489, by virus isolation from blood and they developed antibody responses to dengue virus. PBMC were also obtained from healthy blood bank donors from Massachusetts who did not have detectable levels of antibodies to dengue virus determined using a plaque reduction neutralization test that has been described earlier (8). We define dengue-immune donors as dengue antibody-positive donors, and non-immune donors as dengue anti-

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1. Abbreviations used in this paper: DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; HAI, hemagglutination-inhibiting antibodies; [<sup>3</sup>H]TdR, tritiated thymidine; TW20, Tween 20.

body-negative donors. PBMC were separated by Ficoll-Hypaque density gradient centrifugation methods (9). Cells were resuspended at the concentration of  $1 \times 10^7$ /ml in RPMI 1640 medium containing 10% FCS (Gibco Laboratories, Grand Island, NY) and 10% DMSO (Fisher Scientific Co., Springfield, NJ) and were cryopreserved until use (8).

**Viruses.** The dengue virus strains used were type 1, the Hawaii strain; type 2, the New Guinea C strain; type 3, the CH53489 strain, and type 4, the 814669 strain. Dengue virus types 1 and 2 were supplied by Dr. Walter E. Brandt, and type 4 was supplied by Mr. Jack McCown of the Walter Reed Army Institute of Research. Dengue virus type 3 was supplied by one of the authors (Dr. Bruce Innis).

**Preparation of dengue antigens.** Dengue antigens were prepared using dengue virus-infected Vero cells as previously reported for measles virus (10). Vero cells were infected with dengue virus at an approximate multiplicity of infection of 1 plaque-forming unit/cell, and cultured in MEM containing 2% FCS. When 50% of the monolayer developed cytopathic effects, the cells were removed using cell scrapers (Costar Corp., Cambridge, MA), washed three times with PBS at 4°C, treated with 0.025% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) in PBS for 15 min at 4°C, washed again three times with PBS, and resuspended in RPMI 1640. They were then sonicated with a sonic dismembrator (Fisher Scientific Co., Pittsburgh, PA), and centrifuged at 2,500 rpm for 10 min. The supernatant fluids were collected and used as dengue antigens. Control antigens were prepared in a similar manner using uninfected Vero cells. 3 ml of antigen were obtained from 15 75-cm<sup>2</sup> flasks (Costar Corp.) of confluent Vero cells. Alternative antigens such as purified dengue virus or mosquito cell (C6/36)-derived dengue antigens did not induce specific proliferative responses.

**Induction of proliferative responses of PBMC.**  $2-4 \times 10^5$  PBMC were cultured with dengue antigens diluted at 1:30 in 0.2 ml RPMI containing 10% human AB serum (Hazleton Research Products, Inc., Lenexa, KS) and  $5 \times 10^{-5}$  M 2-mercaptoethanol (Sigma Chemical Co.) in 96-well round-bottom plates (Costar, Cambridge, MA) at 37°C for 6 days. Cells were pulsed with 1.25  $\mu$ Ci of tritiated thymidine ( $[^3\text{H}]\text{TdR}$ ) for 8 h before harvest. Cells were harvested using a multiharvester (Titertek, Skatron Inc., Sterling, VA) and  $[^3\text{H}]\text{TdR}$  incorporation was counted in a liquid scintillation counter (Packard Instrument Co., Sterling, VA).

**Maintenance of dengue antigen-stimulated PBMC.** Bulk cultures were established using  $4 \times 10^5$  PBMC cultured with dengue antigens diluted to a final concentration of 1:30 in 0.2 ml RPMI containing 10% human AB serum, which had been obtained from a healthy, dengue antibody-negative blood bank donor at the University of Massachusetts Medical Center, in 96-well round-bottom plates for 7 d. In some experiments, blast cells were enriched on day 7 by Ficoll-Hypaque density gradient centrifugation.  $2 \times 10^5$  blast cells were cultured with  $2 \times 10^6$   $\gamma$ -irradiated (3,000 rad) autologous PBMC in 2 ml of medium supplemented with 10% IL 2 (Cellular Products, Inc., Buffalo, NY) in 24-well flat-bottom plates (Costar Corp.). On day 14, blast cells were enriched again, and restimulated as described above.

**Establishment of antigen-specific T cell lines using a limited dilution method.**  $4 \times 10^5$  PBMC were cultured with dengue 3 antigen at a final concentration of 1:30 in 0.2 ml RPMI containing 10% human AB serum in 96-well round-bottom plates for 7 d. On day 7, blast cells were enriched by Ficoll-Hypaque density gradient centrifugation and were cultured at concentrations of 30, 10, and 3 cells/well with  $\gamma$ -irradiated autologous PBMC ( $1 \times 10^5$ ) in 0.2 ml RPMI containing 10% human AB serum (University of Massachusetts Medical Center), 10% IL 2, and dengue 3 antigen at a final dilution of 1:30 in 96-well round-bottom plates. On day 14, 0.1 ml of medium was removed from each well and 0.1 ml of fresh medium with human AB serum, IL 2, and dengue antigen was added to maintain the same final concentrations described above. On day 21, cells in wells demonstrating growth were transferred to 48-well flat-bottom plates (Costar Corp.) and were further cultured with  $1 \times 10^6$   $\gamma$ -irradiated autologous PBMC in 1 ml of RPMI containing 10% human AB serum, 10% IL 2, and dengue antigen at a dilution of 1:30.

**Proliferative responses of T cell lines.**  $1 \times 10^4$  T cells were cultured with  $2 \times 10^5$   $\gamma$ -irradiated (3,000 rad) autologous PBMC in 0.2 ml RPMI containing 10% human AB serum and dengue 3 antigen diluted at 1:30 in 96-well round-bottom plates at 37°C for 3 d. Cells were pulsed with 1.25  $\mu$ Ci  $[^3\text{H}]\text{TdR}$  for 8 h before harvest. Cells were harvested using a multiharvester and  $[^3\text{H}]\text{TdR}$  incorporation was counted in a liquid scintillation counter.

**Phenotypic analysis.** Anti-Leu2 (CD8) antibody reacts with suppressor/cytotoxic T cells (11). Anti-Leu3 (CD4) antibody reacts with helper/inducer T cells (11). Anti-Leu4 (CD3) antibody reacts with pan T cells (12). Anti-Leu11 (CD16) antibody reacts with natural killer cells and neutrophils (13). Anti-B1 (CD20) antibody reacts with B cells (14). Anti-Leu2, Leu3, Leu4, and Leu11 antibodies were purchased from Becton-Dickinson & Co. (Mountain View, CA). Anti-B1 antibody was purchased from Coulter Electronics, Inc. (Hialeah, FL). Cell lines and clones were stained with MAb conjugated with fluorescein-isothiocyanate by direct immunofluorescence methods as described earlier (15). They were observed under a fluorescence microscope (Carl Zeiss, Inc., Oberkochen, FRG).

**Immunoassays for IFN.** Sandwich-type ELISA for the estimation of IFN activity were developed at the National Institute for Biological Standards and Control of the United Kingdom. For IFN $\gamma$  determination, 50  $\mu$ l of purified rabbit polyclonal antirecombinant human IFN $\gamma$  (16), diluted 1:200 in PBS, was coated on U-bottomed wells of polyvinyl chloride microtiter plates (Dynatech Laboratories, Alexandria, VA) for 2 h at 37°C. Remaining sites in wells were blocked overnight with 150  $\mu$ l of 2% BSA-PBS at 4°C. Excess antibodies and blocking buffer were removed and wells washed four times with 0.58% Tween 20 (TW20)-PBS. After the last wash, serial dilutions of human IFN $\gamma$  standard (British Standard 82/587, 3,000 IU per ampoule) or culture fluid samples were added, 50  $\mu$ l per well, and incubated at 37°C for 1 h. Wells were then washed four times with TW20-PBS followed by: (a) addition of 50  $\mu$ l purified anti-human IFN $\gamma$  MAb, 4SB3 (17) diluted 1:100 in 2% BSA-PBS, and incubation for 1 h at 37°C; (b) washing with TW20-PBS and addition of 50  $\mu$ l biotinylated sheep anti-mouse immunoglobulin (Amersham International, Amersham, UK), diluted 1:500 in BSA-PBS, and incubation for 1 h at 37°C; (c) washing with TW20-PBS incubation with 50  $\mu$ l streptavidin-biotinylated horseradish peroxidase complex (Amersham International), diluted 1:500 in 2% BSA-PBS and incubation for 30 min at 37°C; and (d) washing twice with TW20-PBS and twice with 0.1 M citrate-phosphate buffer, pH 5.0, followed by the addition of orthophenylene diamine substrate at 1 mg/ml in the 0.1 M citrate-phosphate buffer containing 0.006% hydrogen peroxide. Color was developed in the dark for 30 min at room temperature and the reaction terminated by addition of 50  $\mu$ l 1 M H<sub>2</sub>SO<sub>4</sub> to each well. Optical densities were read at 450 nm in a multiskan (Titertek, Skatron, Inc.). Levels of IFN $\gamma$  in culture fluid samples were interpolated from the IFN $\gamma$  standard calibration curve. The detection limit of the IFN $\gamma$ -specific ELISA was 0.5 IU/ml.

For IFN $\alpha$  determination, the ELISA was carried out in a manner similar to that described above for IFN $\gamma$ -specific ELISA except that the rabbit and mouse antibodies against IFN $\gamma$  were replaced by IFN $\alpha$ -specific antibodies (18, 19). This IFN $\alpha$ -specific ELISA was calibrated with the first international reference preparation for human leukocyte IFN 69/19, 5,000 IU per ampoule, and the detection limit of the assay was 10 IU/ml in this version.

**Effect of IFN produced by PBMC stimulated by dengue antigens on dengue virus infection.** Supernatant fluids from cultures of PBMC of dengue-immune donors that had been stimulated with dengue antigen as described above, were diluted to contain 3 U/ml of IFN $\gamma$ , and culture fluids of the same PBMC exposed to control antigen, which did not contain detectable levels of IFN $\gamma$ , were diluted similarly. These fluids were then used to pretreat human monocytes for 24 h before addition of dengue virus-antibody complexes at multiplicity of infection of 10:1. Previous experiments with recombinant human IFN $\gamma$  pretreatment of monocytic cells had demonstrated that these experimental conditions resulted in the production of a low percentage of infected cells 24 h later in the dengue 2 virus control group (3/212,

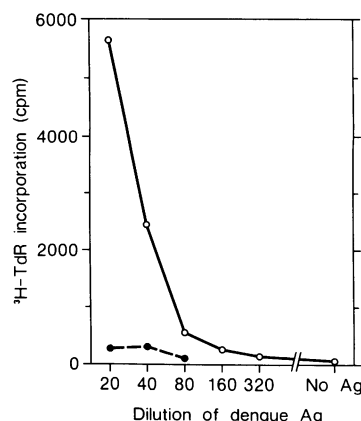
1.4%) and there was no increase in the number of infected cells after pretreatment with IFN $\gamma$ ; however, there were significant increases in the percentage of infected cells after pretreatment with IFN $\gamma$  and exposure to dengue 2 virus-antibody complexes, (29/207, 14% of cells were infected in the absence of IFN $\gamma$ , and 29 and 47%, respectively after pretreatment with 1 or 10 U of IFN $\gamma$ .)

**Statistical analysis.** Differences between values were examined by *t* test and Chi square test. Differences yielding *P* values of < 0.05 were regarded as significant.

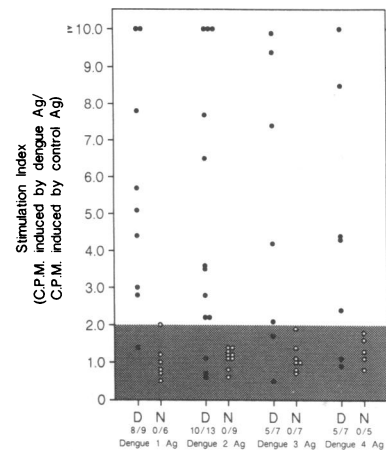
## Results

**Proliferative responses of PBMC from dengue-immune donors induced by dengue antigens.** PBMC from a dengue antibody-positive donor were cultured with dengue or control antigens diluted at various concentrations, and [ $^3$ H]TdR incorporation was examined. Dengue antigens induced significant proliferative responses of PBMC, and there was a good correlation between the level of the proliferative responses and the concentration of dengue antigens (Fig. 1). Control antigen did not induce significant proliferative responses. A time course study showed that the proliferative responses reached maximum levels on day 6 (data not presented).

The results shown in Fig. 2 illustrate the proliferative responses of PBMC obtained from 11 Thai donors and the 2 American donors who were known to have previously been infected with dengue virus. The PBMC from these donors showed significant proliferative responses induced by dengue antigens (8 out of 9 with dengue 1 Ag; 10 out of 13 with dengue 2 Ag; 5 out of 7 with dengue 3 Ag; 5 out of 7 with dengue 4 Ag). The PBMC from Massachusetts blood bank donors did not significantly proliferate in response to dengue antigens. The failure of PBMC of some Thai donors to respond to certain dengue antigens may be partly because the PBMC were derived from healthy adults, and dengue infections are most common during childhood. Table I contains a summary of the positive proliferative responses induced by dengue antigens using PBMC from Thai and American donors. These results indicate that antigens of the four dengue serotypes can induce proliferative responses using PBMC from dengue antibody-positive donors. Neither dengue antigens nor control antigens induced significant proliferative responses of PBMC from nonimmune donors.



**Figure 1.** Proliferative responses of dengue-immune PBMC to dengue Ag.  $2 \times 10^5$  PBMC from an American donor who had been infected with dengue 3 virus were cultured with dengue 3 Ag or control Vero Ag at various dilutions for 6 d. Cells were pulsed with  $1.25 \mu\text{Ci } ^3\text{H-TdR}$  for 8 h before being harvested. ○, proliferative responses induced by dengue 3 antigen. ●, proliferative responses induced by control antigen.



**Figure 2.** Proliferative responses of PBMC from 11 Thai and 2 American antibody-positive donors.  $4 \times 10^5$  PBMC were cultured with dengue and control Vero antigens diluted at 1:30 for 6 d. Cells were pulsed with  $1.25 \mu\text{Ci } [^3\text{H}]\text{TdR}$  for 8 h before harvest. Results were expressed as a stimulation index that was calculated as follows: mean counts per minute induced by dengue Ag/mean counts per minute induced by control Ag. Stimulation indices > 2 were considered as positive proliferative responses. D, dengue-immune donors. N, nonimmune donors.

**Detection of IFN $\gamma$  in the culture fluids of PBMC stimulated with dengue antigens.** We examined the culture fluids of PBMC stimulated with dengue antigens for IFN $\gamma$  using ELISA with MAb to human IFN $\alpha$  and  $\gamma$ . IFN $\gamma$  was detected at high titer in the culture fluids of PBMC from dengue-immune donors stimulated with dengue antigens (*P* < 0.02 compared with the amount of IFN $\gamma$ -detected in cultures of PBMC from nonimmune donors), but not in the culture fluids of PBMC stimulated with control antigen or cultured alone (Table II). IFN $\alpha$  was not detected in the culture fluid of PBMC after stimulation with dengue or control antigen (data not presented). The culture fluids of PBMC from antibody-negative donors stimulated with dengue antigens did not contain significant titers of IFNs. These results indicate that dengue-immune PBMC produce high titers of IFN $\gamma$  after stimulation with dengue antigens, but do not produce IFN $\alpha$ . IFN $\gamma$  was detected at low titers in the culture fluids of PBMC from some donors cultured without antigens. It has been reported that PBMC from some normal adults produce IFN $\gamma$  during in vitro culture without the addition of specific antigens (20).

**Characterization of the lymphocytes responding to dengue antigens.** We cultured the proliferating lymphocytes with  $\gamma$ -irradiated autologous PBMC in the presence of dengue antigens and IL 2 for 7 or 14 d, after the original 7 d of bulk culture stimulation, to characterize the lymphocytes that responded to dengue antigen. Phenotypic analyses showed that the proliferating cells were predominantly CD3+, CD4+, CD8-, CD16-, and CD20- (Table III). Therefore, the proliferating cells are T cells with the helper/inducer phenotype. They produced IFN $\gamma$  after stimulation with dengue antigens, but not after stimulation with control antigen (Table IV).

**Analysis of responding cells by establishing dengue-specific T cell lines.** We further characterized the T cells that respond to dengue antigen by establishing dengue-specific T cell lines by a limiting dilution method using lymphocytes from one American donor who was known to have been infected with dengue 3 virus. 14 T cell lines, which responded to dengue 3 antigen but not to control antigen, were established. Three of these lines were further expanded. The JK3 and JK10 lines were established from wells containing 10 blast cells. The JK15 line was established from a well containing 30 blast cells. The

Table I. A Summary of Proliferative Responses of PBMC from Dengue-immune Donors to Dengue Antigens

Dengue-immune*				Nonimmune		
		[3H]TdR incorporation				[3H]TdR incorporation
No. of donors		Stimulation index		No. of donors	Stimulation index	
			<i>cpm</i>	<i>cpm</i>		
Dengue 1	8	7.4 (2.8–15.7)	9,011 (1,018–19,343)	6	1.0 (0.5–2.0)	664 (142–2,357)
Dengue 2	10	7.5 (2.2–19.5)	4,644 (1,019–10,872)	9	1.1 (0.6–1.4)	1,038 (93–2,897)
Dengue 3	5	6.6 (2.1–9.9)	6,005 (1,706–13,762)	7	1.1 (0.7–1.9)	732 (158–1,975)
Dengue 4	5	8.2 (2.4–21.5)	7,936 (2,153–17,178)	5	1.3 (0.8–1.8)	611 (185–1,275)
Control Ag	11	—	1,046 (68–4,033)	9	—	783 (261–2,172)
No Ag	11	—	630 (69–1,980)	9	—	529 (95–1,723)

$4 \times 10^5$  PBMC were cultured for 6 d with dengue or control Ag diluted at 1:30. Cells were pulsed with 1.25  $\mu$ Ci [<sup>3</sup>H]TdR for 8 h before harvest. Results are presented as averages. \* The responses of PBMC of dengue-immune donors who had a stimulation index greater than two with each of the four dengue antigens are included. The stimulation index was calculated from mean counts per minute induced by dengue Ag/mean counts per minute induced by control Ag. Quadruplicate samples were used.

cloning efficiency was 23% with 10 cells/well, and 56% with 30 cells/well, respectively. All three lines had CD3+, CD4+, and CD8– phenotypes (data not presented), and responded to dengue 3 antigen, but did not respond to control antigen (Table V). They produced IFN $\gamma$  after stimulation with dengue 3 antigen but did not produce IFN $\gamma$  after stimulation with control antigen (Table V). They did not produce significant titers of IFN $\alpha$ .

Table II. IFN $\gamma$  Production by PBMC from Dengue Antibody-Positive Donors after Stimulation with Dengue Antigens

		IFN $\gamma$ *				
Donors	Dengue 1 Ag	Dengue 2 Ag	Dengue 3 Ag	Dengue 4 Ag	Control Ag	No Ag
<i>U/ml</i>						
Dengue antibody-positive						
T1	25	120	28	70	2	2
T2	42	56	51	115	14	14
T3	33	47	14	26	1	3
A1	32	43	61	38	3	3
Dengue antibody-negative						
M1	8	5	8	5	5	6
M2	3	11	4	3	2	3
M3	<1	2	2	2	2	2
M4	<1	<1	<1	<1	<1	<1

$4 \times 10^5$  PBMC were cultured for 6 d with dengue and control Ag diluted at 1:30. Culture fluids were examined for IFN $\gamma$  by ELISA.

\* The titers of IFN $\gamma$  induced by dengue and control antigens were compared by *t* test between dengue antibody-positive and antibody-negative donors. IFN $\gamma$  induced by dengue 1 Ag,  $P < 0.001$ ; by dengue 2 Ag,  $P < 0.02$ ; by dengue 3 Ag,  $P < 0.02$ ; by dengue 4 Ag,  $P < 0.01$ ; by control Ag,  $P < 0.2$  (not significant); without Ag,  $P > 0.2$  (not significant).

Augmentation of dengue virus infection of human monocytes by IFN $\gamma$  produced by PBMC in response to dengue antigen. We tried to determine whether the culture fluids of PBMC stimulated with dengue antigen could augment dengue virus infection of human monocytes. We recently reported that recombinant human IFN $\gamma$  augmented dengue virus infection of human monocytes and monocytic cells in the presence of anti-dengue antibody (5). Culture fluids were obtained from dengue-immune PBMC after stimulation with dengue antigen, and they were then diluted to contain 3 U/ml of IFN $\gamma$ . Culture

Table III. Phenotypes of Short-Term-cultured T Cell Lines Stimulated with Dengue Antigens

		Days of culture	% positive cells*				
Donor	Ag		CD3	CD4	CD8	CD16	CD20
A1	Dengue 3	14	89	84	2	2	<1
		21	91	83	3	ND	ND
T3	Dengue 2	14	74	46	24	ND	ND
T4	Dengue 2	14	84	80	8	ND	ND

$4 \times 10^5$  PBMC were cultured with dengue antigens diluted at 1:30 in 0.2 ml RPMI 1640 containing 10% human AB serum in 96-well round-bottom plates for 7 d. On day 7, blast cells were enriched by Ficoll-Hypaque density gradient centrifugation.  $2 \times 10^5$  blast cells were cultured with  $2 \times 10^6$   $\gamma$ -irradiated autologous PBMC in 2 ml RPMI 1640 containing 10% human AB serum, 10% IL 2 and dengue antigens at a final dilution of 1:30 in 24-well flat-bottom plates for 7 d, and phenotypes were examined. One cell line was restimulated again on day 14 and phenotypes were examined on day 21.

\* Mab used were anti-Leu4 Ab for CD3, anti-Leu3 Ab for CD4, anti-Leu2 Ab for CD8, anti-Leu11a Ab for CD16, and anti-B1 Ab for CD20.

Table IV. Proliferative Responses and IFN $\gamma$  Production by a Short-Term-cultured T Cell Line after Stimulation with Dengue Antigens

Culture days	<sup>3</sup> H-TdR incorporation			IFN					
	Dengue Ag	Control Ag	No Ag	Dengue Ag		Control Ag		No Ag	
				IFN $\gamma$	IFN $\alpha$	IFN $\gamma$	IFN $\alpha$	IFN $\gamma$	IFN $\alpha$
cpm			U/ml						
14	1,325	250	270	40	<10	<1	<10	<1	<10
21	1,290	380	432	280	<10	12	<10	10	<10

$5 \times 10^4$  short-term-cultured T cells were cultured with  $2.5 \times 10^5$   $\gamma$ -irradiated autologous PBMC in 0.2 ml RPMI 1640 containing dengue 3 Ag or control Ag diluted at 1:30 for 3 d. Cells were pulsed with 1.25  $\mu\text{Ci}$  [ $^3\text{H}$ ]TdR for 8 h before harvest. Culture fluids were examined for IFN $\gamma$  and IFN $\alpha$  by ELISA.

fluids of the same dengue-immune PBMC that had been exposed to control antigen were similarly diluted. Human monocytes were exposed to these culture fluids for 24 h. They were then washed and infected with dengue virus-antibody complexes. A similar percent of the monocytes that were cultured without the addition of culture fluids from PBMC and that were treated with the diluted culture fluid from dengue-immune PBMC that had been exposed to control antigen became infected and expressed dengue antigens; however, increased number of the monocytes treated with diluted culture fluids from dengue-immune PBMC that had been exposed to dengue antigen and contained 3 U/ml of IFN $\gamma$  had dengue viral antigens (Table VI,  $P < 0.001$  in exp. 1 and  $P < 0.01$  in exp. 2). This augmenting effect of dengue antigen-stimulated culture fluid was abrogated by anti-IFN $\gamma$  antibody, but not by anti-IFN $\alpha$  antibody (data not presented). These results confirmed our previous report that IFN $\gamma$  augments dengue virus infection of human monocytes in the presence of anti-dengue antibody, and suggests that dengue-specific T lymphocytes may have an important role in the pathogenesis of secondary dengue virus infections by increasing the number of dengue virus-infected monocytes via IFN $\gamma$  production.

## Discussion

The role of T lymphocytes in recovery from dengue virus infections and in the pathogenesis of severe complications has not been explained. In this paper, we demonstrated dengue-

specific T lymphocyte proliferative responses with PBMC of dengue antibody-positive humans. We used fixed, sonicated dengue virus-infected Vero cells as antigens, as previously were reported with measles virus (10), because cell-free preparations of live dengue virus did not stimulate PBMC of dengue-immune donors (data not presented). Proliferative responses induced by these antigens are dengue specific, because PBMC of dengue antibody-positive donors respond to dengue antigens but not to control antigen, and PBMC of antibody-negative donors do not respond to dengue or control antigens. Antigens of the four dengue serotypes induced generally similar levels of proliferative responses of PBMC from the Thai adult donors. The dengue infection histories of these adult Thai donors are not known, but they all have high levels of antibodies to the four dengue serotypes, presumably as a result of prior natural infections. The PBMC of an American donor, who had been immunized with yellow fever vaccine and later became infected with dengue 3 virus, responded best to dengue 3 antigen but also responded to dengue 1, 2, and 4 antigens to some degree (data not presented). These results suggest that human T cell responses to primary dengue infections include both type-specific and serotype cross-reactive responses. Further studies are in progress to determine the subtype specificities of human T cell responses to dengue infections.

The dengue-specific proliferating lymphocytes are T cells with CD4 phenotype. These results were obtained using bulk culture-stimulated PBMC and dengue-specific T cell lines. It has been reported that CD4 $^+$  T cells can be divided into two

Table V. Proliferative Responses and IFN $\gamma$  Production by Dengue-Specific T Cell Lines after Stimulation with Dengue Antigens

Lines	<sup>3</sup> H]TdR incorporation			IFN					
				Dengue Ag		Control Ag		No Ag	
	Dengue Ag	Control Ag	No Ag	IFN $\gamma$	IFN $\alpha$	IFN $\gamma$	IFN $\alpha$	IFN $\gamma$	IFN $\alpha$
	cpm			U/ml					
JK3	6,185	514	345	70	22	2	<20	<1	21
JK10	1,978	407	275	44	<20	4	<20	2	<20
JK15	3,737	471	206	44	<20	4	<20	3	<20

$1 \times 10^4$  cells were cultured with  $2 \times 10^5$   $\gamma$ -irradiated autologous PBMC in 0.2 ml RPMI 1640 containing dengue 3 Ag or control Ag diluted at 1:30 for 3 days. Cells were pulsed with 1.25  $\mu\text{Ci}$  [ $^3\text{H}$ ]TdR for 8 h before harvest. Culture fluids were examined for IFN $\gamma$  and IFN $\alpha$  by ELISA.

**Table VI. Augmentation of Dengue Virus Infection of Human Monocytes by IFN $\gamma$  Produced by PBMC in Response to Dengue Antigen**

Treatment of monocytes*	% dengue antigen-positive cells	
	Exp. 1	Exp. 2
None (RPMI 1640 containing 10% FCS)	22% (64/286)	5% (15/274)
Diluted culture fluid of PBMC stimulated with dengue antigen	37% (99/266) <sup>§</sup>	12% (38/325) <sup>†</sup>
Diluted culture fluid of PBMC stimulated with control antigen	24% (75/310) <sup>  </sup>	5% (14/301) <sup>‡</sup>

The percentage of dengue antigen-positive monocytes pretreated with culture fluids of PBMC stimulated with dengue antigen or control antigen were compared with the percentage of dengue antigen-positive monocytes which was not treated with culture fluid by Chi-square test. <sup>‡</sup> $P > 0.7$  (NS); <sup>§</sup> $P < 0.001$ ; <sup>||</sup> $P < 0.5$  (NS); <sup>†</sup> $P < 0.01$ .

\* Culture fluids obtained from dengue-immune PBMC after stimulation with dengue antigen were diluted to contain 3 U/ml of IFN $\gamma$ , and culture fluids of the same PBMC exposed to control antigen, which did not contain detectable levels of IFN, were diluted similarly. After treatment with these fluids for 24 h, human monocytes were infected with dengue virus or dengue virus-antibody complexes as described in Methods. The percentage of monocytes infected with dengue virus was determined 24 h after infection by indirect immunofluorescence.

subsets, helper T cells for antibody production (21) and the inducer of the CD8<sup>+</sup> suppressor cells (22). It has also been reported that virus-specific, HLA class II-restricted cytotoxic T cell clones have the CD4 phenotype (23–25). The biological functions of dengue-specific CD4<sup>+</sup> T cells will be analyzed in the future experiments. We are actively pursuing the analysis of dengue-induced T cell responses at the clonal level and have detected dengue specific cytotoxicity (data not presented). Our results, which demonstrate that CD4<sup>+</sup> T cells respond to dengue-Vero antigen, do not rule out a contribution for CD8<sup>+</sup> T cells during dengue virus infections *in vivo*. It has been reported that the phenotypes and functions of T cells generated *in vitro* after stimulation with viral antigens may vary, depending on the form of the viral antigens used to stimulate, in the case of human cytomegalovirus (26) and varicella zoster virus (27). Those reports state that cell-free virus preferentially induces the proliferation of CD4<sup>+</sup> T cells, and that virus-infected autologous fibroblasts stimulated cytotoxic T lymphocytes with a CD8<sup>+</sup> phenotype (26, 27). We will examine the T cell response at the clonal level, and use autologous cell types as alternative antigen-presenting cells in search for CD8<sup>+</sup> responders.

Through the application of these techniques, we hope to define the dengue epitopes which are recognized by human T lymphocytes. Dengue virus has three structural proteins; envelope protein (E), membrane protein (M), and capsid protein (C) (28, 29). It also induces seven nonstructural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (28, 30). Although the role and immunogenic properties of these 10 proteins has not been defined, E protein is thought to contain epitopes that induce neutralizing antibody responses (31). MAb to E protein protect mice from lethal dengue infection (32). Recently, it has been reported that immunization of mice with dengue 2 virus

NS1 protein provided significant protection against lethal challenge with dengue 2 virus (33). Identification of the epitopes or dengue antigens that are recognized by T cells and may have a role in protection or in the pathogenesis of DHF and DSS is an important area for research.

The severe complications of dengue virus infections, DHF and DSS, are much more commonly observed during secondary dengue infections than during primary infections (1, 2, 34). However, a small percentage of DHF/DSS cases is observed during primary infections and most of these are primary infections of infants between 6 and 12 mo of age born to dengue antibody-positive mothers (1, 2). Kliks et al. recently reported that there is a correlation between the level of maternal dengue antibodies and occurrence of DHF/DSS of the infants during the first year of age (35). Therefore, although we speculate that there is a role for dengue-specific T cells in the pathogenesis of DHF/DSS, which is much more commonly observed during secondary infections, such specific T cell memory responses do not explain these complications when they occur, although much less frequently, during primary infections.

In this paper, we demonstrated that dengue-specific CD4<sup>+</sup> T cells produce high titers of IFN $\gamma$ . It has been reported that antigen-specific T cells produce IFN $\gamma$  after stimulation with viral antigens (6, 36, 37). The role of IFN $\gamma$  produced by dengue-specific T lymphocytes is another important subject to be further elucidated. IFN $\gamma$  has potent immunoregulatory functions (38, 39). IFN $\gamma$  increases Fc $\gamma$  receptors on human monocytes (40, 41). Human monocytes have been identified as cells that support dengue virus infections (42). It has been reported that anti-dengue antibodies at subneutralizing concentrations augment dengue virus infection of human monocytes, secondary to the increased uptake by monocytes of dengue virus in the form of dengue virus-antibody complexes via Fc receptors (3). We recently reported that recombinant human IFN $\gamma$  augments dengue virus infection of human monocytes and the human monocytic cell line U937 in the presence of anti-dengue antibody, and that the augmented dengue virus infection is due to the augmented expression of Fc receptors induced by IFN (5). In these experiments, we showed that dengue virus-specific CD4<sup>+</sup> T cells produce high levels of IFN $\gamma$  in response to dengue antigens and that culture fluids which contain IFN $\gamma$  produced by dengue-specific T cells augment dengue virus infection of human monocytes in the presence of anti-dengue antibody. T cell sensitization and IFN $\gamma$  production would occur during primary dengue infections, and the restimulation of primed cross-reactive T cells, production of IFN $\gamma$  and the presence of virus-antibody complexes in the early phases of secondary dengue infections suggest a model for the pathogenesis of DHF/DSS. Based on these observations, we hypothesize that: IFN $\gamma$  is produced by dengue-specific T lymphocytes after stimulation by dengue virus antigens, and the produced IFN $\gamma$  might contribute to the pathogenesis of DHF and DSS because IFN $\gamma$  increases Fc $\gamma$  receptors on human monocytes and this increases the number of dengue virus-infected monocytes in the presence of anti-dengue antibodies during secondary dengue infections, and by activating macrophages to release vasoactive compounds.

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